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Award Number: **W81XWH-12-1-0331**

TITLE: **Apoptosis Induction by Targeting Interferon Gamma Receptor 2 (IFNgammaR2) in Prostate Cancer: Ligand (IFNgamma)-Independent Novel Function of IFNgammaR2 as a Bax Inhibitor**

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REPORT DATE: August 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE
August 2013

2. REPORT TYPE
Annual

3. DATES COVERED
1 August 2012-31 July 2013

4. TITLE AND SUBTITLE
Apoptosis Induction by Targeting Interferon Gamma Receptor 2 (IFNgammaR2) in Prostate Cancer: Ligand (IFNgamma)-Independent Novel Function of IFNgammaR2 as a Bax Inhibitor

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0331

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER
Task 1

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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Cleveland, OH, 44106

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In our previous study, we found that IFNγR2 has previously unknown function as an inhibitor of Bax. Bax is a key mediator of apoptosis. We found that IFNγR2 is overexpressed in prostate cancer, and we hypothesize that abnormally high level of IFNγR2 confers apoptosis resistance of prostate cancer. In this project, we will investigate the role of IFNγR2 in drug resistance of prostate cancer and explore the development of therapeutic peptide that can activate Bax-induced apoptosis in prostate cancer by inactivating IFNγR2. In the first 12 months (Year 1), we planned to perform experiments investigating the binding domains of IFNγR2 and Bax. We generated plasmids encoding mutant cDNAs of Bax and IFNγR2 in which previously known functional domains are deleted. These mutant proteins were expressed in the cell, and their Bax inhibition activity and binding activities were analyzed. As a result, we found that the IFNγR2 binding domain of Bax is localized to amino acids 54-198 of Bax, and Bax binding domain of IFNγR2 is localized to amino acids 296-337 of IFNγR2. We will perform further investigation to identify minimum essential binding domains in IFNγR2 and Bax. These studies will help us to design therapeutic peptide that can disrupt Bax-IFNγR2 interaction to promote Bax-induce apoptosis triggered by chemotherapy in prostate cancer.

15. SUBJECT TERMS
None provided

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU		

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Introduction

In our preliminary study, we identified interferon g receptor 2 (IFNγR2)as a Bax suppressor using yeast-based functional screening of Bax inhibiting proteins. Bax is a key mediator of apoptosis which is essential for chemotherapy-induced apoptosis of prostate cancer cells. We found that IFNγR2 levels is abnormally elevated in prostate cancer cell lines (both androgen-dependent and –independent cell lines). shRNA-mediated knockdown of IFNγR2 was able to increase chemotherapy-induced apoptosis rate significantly in prostate cancer cells, suggesting that IFNγR2 is an chemo-resistant factor in prostate cancer cells. Although IFNγR2 was previously known as a receptor of IFNγ which is an anti-tumorigenic cytokine, our preliminary data suggest that IFNγR2 expresses its anti-apoptosis (anti-Bax) activity independent from IFNγ and IFNγ signaling. Importantly, we found that IFNγR2 is expressed in mitochondrial membranes and ER membranes, but not on the plasma membranes of prostate cancer cells. Since we found that IFNγR2 can directly interact with Bax in vitro, we hypothesize that IFNγR2 confer apoptosis resistance of prostate cancer by directly binding and inhibiting Bax.

In this 3 years DOD Prostate Cancer Research IDEA project, the following Tasks will be examined to develop novel anti-prostate cancer therapy as well as to establish IFNγR2 as a diagnostic maker to predict the chemo-resistance of prostate cancer.

Task 1: To determine the mechanism of Bax inhibition by IFNγR2, and to develop anti-IFNγR2 peptide that enhances Bax activation. (Months 1-24)

Task 2: To identify the subtype of prostate cancer that can be effectively treated by IFNγR2-targeting technologies (Months 13-36)

Task 3: Determination of the mechanism of abnormal expression of IFNγR2 in prostate cancer (Months 13-36)

In the first year, we planned to perform (1) generation of plasmids that express mutant proteins of Bax or IFNγR2 lacking possible binding domains, and (2) examination of the activities of Bax inhibition and binding of these mutant proteins. These experiments will provide necessary information to design IFNγR2 inhibiting peptides that will promote Bax-induced cell death in prostate cancer.

Body (Methods, Results and Discussion)

Results and Discussion

<u>Task 1a: Search for IFNγR2 binding domain of Bax</u>

1. We made a series of plasmids that express Bax mutant proteins lacking possible IFNγR2 binding domains. Bax mutant cDNAs were subcloned into Flag-tagged and GFP-tagged protein expression plasmids.

Strategy of making Bax mutants:
Human Bax protein has 198 amino acids. There are 9 alpha helices. The first N-terminal 53 amino acids before the 1st alpha helix is known to function as an auto-inhibitory domain. The 1st alpha helix is also known as BH3 domain that is essential to bind other Bcl-2 family proteins (including Bax itself). Alpha helices 5 and 6 creates channels in the membrane that are required for pore-forming activity of Bax, and this activity is implicated to be essential for cytochrome c release from mitochondria. Alpha helix 9 is a trans-membrane domain that is essential for mitochondrial membrane localization of Bax,

and this domain is also essential for apoptosis induction. Each of these domains essential for Bax-mediated apoptosis was deleted, and fused with epitope tag (Flag- or GFP-tag) so that we can detect expression of these Bax mutants by antibodies detecting epitope tags. Expression of these proteins in HEK293T cells have been confirmed (we will confirm the expression in PC3 soon).

List of newly made plasmid:

*pCMV2B vector express Flag-tagged protein. pEGFP vector express EGFP-tagged protein. Bax full length cDNA has been already subcloned into these vectors.

pCMV2B-Bax ΔN (N-terminal 53 amino acid was deleted)
pCMV2B-Bax Δα1 (a-helix 1 is deleted)
pCMV2B-Bax Δα 5-6 (a5 and a6 are deleted)
pcMV2B-Bax Δα 9 (a9 is deleted)
pEGFP-Bax ΔN
pEGFP-Bax Δα 1
pEGFP-Bax Δα 5-6
pEGFP-Bax Δα 9

As explained from the next section, these plasmids were used for the search of IFNγR2 binding domain of Bax.

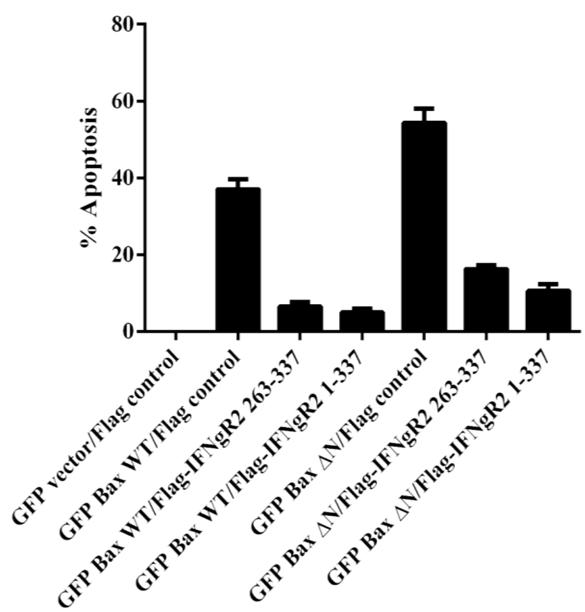


Fig.1. The N-terminal 53 amino acids of Bax is not required for Bax inhibition by IFNγR2. Bax or Bax without 1-53 amino acids (Bax ΔN) were expressed as GFP-fusion protein together with Flag-tagged IFNγR2 (full length) or –IFNγR2 (263-337). GFP vector or Flag-tagged control protein (firefly luciferase) were used as a negative control. IFNγR2 and IFNγR2 (263-337) were able to inhibit apoptosis induced by both Bax and Bax ΔN, suggesting that the first 53 amino acids of Bax is not the target of IFNγR2 to inhibit Bax-induced apoptosis.

2. We found that the first 53 amino acid of Bax is not required for IFNγR2 to inhibit Bax.

The first N-terminal 53 amino acids is known to be auto-inhibitory domain that keeps Bax in the cytosol. If this domain is cleaved, Bax is known to be hyperactive to induce apoptosis. The mechanism by which this N-terminal domain suppresses activation of Bax is not very well known.

We speculated that IFNγR2 binds to this N-terminal domain to stabilize inactive status of Bax. If it is the case, IFNγR2 will not be able to suppress apoptosis induced by Bax ΔN (1-53 amino acids are deleted). To examine this speculation, we performed co-transfection of GFP- Bax ΔN and Flag-tagged IFNγR2 in HEK293 cells (Fig.1).

The conclusion is that IFNγR2 (both full length and C-terminal 41 amino acids) was able to suppress Bax ΔN –induced apoptosis. This result suggests that IFNγR2 interacts with unidentified domain in the region of amino acids 54-198 (next section explains the strategy to narrow down the binding domain within this

region). To further confirm that IFNγR2 can inhibit hyperactive- Bax ΔN even in prostate cancer cells, we will perform the similar experiments in PC3 cell lines.

3. Binding experiments using co-immunoprecipitation

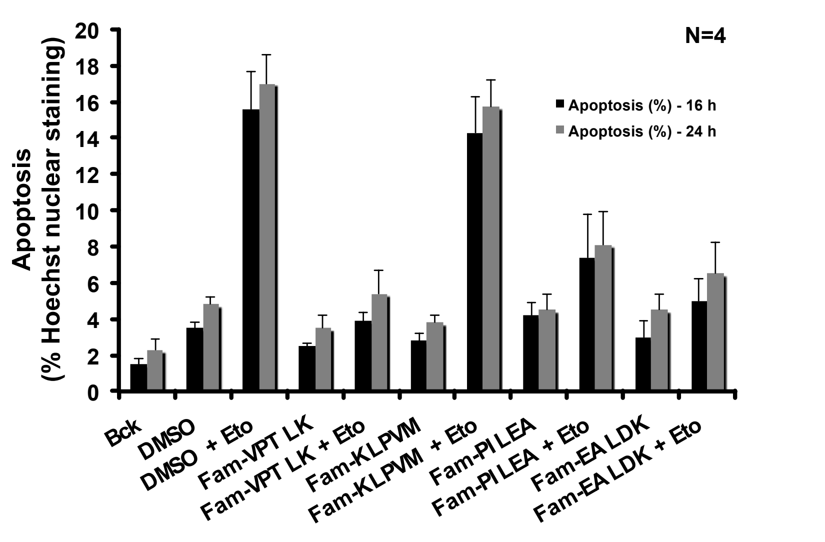
Using GFP-Bax plasmids prepared in section 1, we performed co-immunoprecipitation (co-ip) experiments to identify essential domain for the binding of IFNγR2. IFNγR2 was expressed as Flag-tagged protein so that we can detect co-immunoprecipitated IFNγR2 by Flag-antibody. However, we faced a problem. Molecular Weight (MW) of Flag-IFNγR2 is approximately 52 kDa, and it is very close to 50 kDa IgG Heavy Chain. Unfortunately the 2nd antibody used to detect Flag-tagged IFNγR2 cross reacted with IgG of anti-GFP antibody that was used to pull down GFP-Bax-Flag-IFNγR2 complex, and IgG signal interfered the signals from 52 kDa IFNγR2. In general, to avoid this type of the problem, antibodies from different animal (e.g. the combination of rabbit IgG and mouse IgG) are used for co-ip and Western blot detection. In our experiment, we used rabbit IgG for GFP antibody and mouse IgG for Flag-IFNγR2 detection, and the 2nd antibody to detect Flag-IFNγR2 was anti-mouse IgG, but not anti-rabbit IgG. But, still, there was a cross reactivity. To solve this problem, we are now examining alternative methods to detect Flag-IFNγR2 signal.

We made IFNγR2 antibody conjugated with HRP so that we can detect IFNγR2 directly without 2nd antibody. Theoretically, we will be able to detect Flag-IFNγR2 signal without the interference from IgG used for co-ip. We are now optimizing the condition. In addition, we will perform co-ip by pulling down Flag-antibody (purified monoclonal antibody). In this case, we need to detect Bax by GFP antibody. Molecular weight of GFP-Bax is also close to 50 kDa, unfortunately. However, we have more choices of 2nd antibodies to avoid cross reactivity with mouse monoclonal antibody. We are also planning to perform alternative strategy in which IgG against Bax or IFNγR2 will be covalently conjugated to sepharose beads for immunoprecipitation. If this method works, we will be able to minimize the contamination of IgG in Western blot analysis.

<u>Task 1b: Search for Bax binding domain of IFNγR2</u>

1. Bax binding domain of IFNγR2 is located in amino acids 296-337 (41 amino acids), and the minimum essential domain may exist in amino acids 301-308.

Fig.2 IFNgR2 derived penta-peptides partially protected HeLa cells against etoposide-induced apoptosis.



IFNγR2 has 337 amino acids. In our preliminary study shown in our proposal, we found that amino acids 296-337 of IFNγR2 (IFNγR2 (296-337)) are sufficient to bind Bax and inhibit Bax-induced apoptosis. Fig.2 shows that recombinant protein of IFNγR2 (296-337) was able to bind Bax. In addition, we observed that expression of GFP-tagged IFNγR2 (296-337), but not IFNγR2 (1-295) suppressed Bax-overexpression-induced apoptosis. These results suggest that Bax binding domain of IFNγR2 is located in 296-337 (41 amino acids).

Among 41 amino acids in IFNγR2 (296-337), there is an amino acid sequence of PILEADK (amino acids 301-308) which has a similarity to the Bax binding domain of Ku70 (Ku70 is an Bax inhibiting protein) that was discovered by PI's group. Ku70's Bax binding domain is (V)PTLKEA. We examined whether this PILEADK contains Bax inhibiting activity by examining its effects on Bax-mediated cell death (Fig.2), and we observed that penta-peptides (PILEA and EALDK) designed from this sequence has a weak apoptosis inhibition activity. Ku70 derived Bax inhibiting peptide (VPTLK) was used as a positive control for Bax-mediated cell death inhibition. KLPVM is a negative control penta-peptide that does not have Bax inhibiting activity. In the preliminary data shown in Fig.2, we used Etoposide (10 uM 16hs and 24 hrs treatment)-induced apoptosis in HeLa cells that is known to be induced by Bax-dependent cell death pathway. All the peptides used in this experiments were taged with fluorescent tag (Fam) so that peptide entry into the cells can be monitored and confirmed. All the peptides tagged with Fam were able to enter the cell in this experiment. We will re-examine anti-apoptotic activity of PILEA and EALDK using prostate cancer cells (PC3) using both docetaxel and Bax-overexpression as inducers of Bax-induced apoptosis.

We will continue experiments to confirm Bax inhibiting activity of these peptides by examining more thorough dose-response experiments. In addition, we will examine whether these peptides had Bax binding activity. To examine binding, biotin-labeled peptide will be incubated with Bax in vitro, and the complex will be pulled down by strept-avidin beads.

3. Generation of deletion mutant to search Bax binding domain in IFNγR2.

We are also planning to generate deletion mutant IFNγR2 cDNA lacking small portion (such as 10 amini acids) in the C-terminal 41 amino acids that contain Bax binding domain. These deletion mutants will be generated by 2-step PCR based mutagenesis, and these cDNA will be subcloned into pEGFP (GFP-tag) or pCMV (Flag-tag) vector. Then, co-immunoprecipitation with Bax will be examined to identify Bax binding domain(s).

Methods:

Cell culture and plasmid transfection:
HEK293T cells and PC3 cells were obtained from ATCC, and these cells were cultured in DMEM containing 10%FCS and1% penicillin/streptomycin. For plasmid transfection to examine Bax inhibiting activity and Bax binding activity of IFNγR2, $2x10^5$ cells/6 cm dish and $1x10^6$ cells/10 cm dish, respectively, were used. Plasmid transfection were performed by using Superfect (Qiagen). For bax inhibiting activity, 0.5 ug Bax expressing plasmid and 2.0 ug IFNγR2 expressing plasmids were co-transfected to 6 cm dish culture. For Bax binding experiments (co-immunoprecipitation experiments), 1 ug of Bax expressing plasmids and 4 ug of IFNγR2 expressing plasmids were transfected to the cells cultured in 10 cm dish. 24 hrs after the transfection, cells were collected for apoptosis analysis or cell lysate preparation for binding experiments as described below.

Generation of Plasmid:
pCMV vector and pEGFP vectors were purchased from Clontech. Bax mutant cDNAs were generated by 2 step PCR methods, and these mutant cDNAs were subcloned into pCMV and pEGFP vectors.

Peptide Synthesis:
Peptides were purchased from Biopeptide (San Diego) by order made. Peptides were purified by HPLC and the purity is more than 99% so that possibly toxic chemicals were removed fro the peptide solution. Peptides were dissolved in PBS at the concentration of 100 mM, and 200 uM were added to the cell culture to examine whether the peptide can inhibit Bax-mediated cell death.

Cell Death Assay:

After 24 hrs of plasmid transfection, cells were resuspended in HBSS (Hanks Buffered Saline Solution) at 4C, and Hochst 33258 (DNA staining fluorescent dye, purchased from Sigma) were added at 10 ug/ml concentration. Under fluorescent microscope, transfected cells were identified by GFP signal, and percentages of apoptotic cells showing apoptotic nuclear fragmentation or condensation among GFP positive cells were measured. Total 300 cells were counted in each sample.

Binding experiments:
Cells were lysed by lysis buffer (1% NP40 10 mM HEPES and 140 mM NaCl) containing protease inhibitor cocktail (purchased from Sigma). Cell lysates are adjusted to 2.5 ug/ul protein concentration by the lysis buffer. 200 ul of cell lysates were precleaned by shepharose beads, and the cleaned lysates were subjected to co-immunoprecipitation using anti-GFP antibody. After 2 hrs of incubation with GFP antibody, immunocomplex were pulled down by Protein A coated Sepharose beads (Pharmacia). After 3 times wash of the beads by lysis buffer, immunocomplex was eluted by 50ul of Lamli buffer for SDS-PAGE. Twenty ul of sample were applied to each lane of SDS-PAGE and Western blot was performed by using anti-flag antibody.

Key Research Accomplishment

1. Plasmids that express GFP- and Flag-tagged Bax mutant proteins were prepared.
2. We found that The N-terminus of Bax is not essential for IFNγR2 to inhibit Bax-induced apoptosis.
3. We confirmed that Bax binding domain of IFNγR2 is localized in the C-terminal 41 amino acids of IFNγR2 (IFNγR2 (263-337) was able to inhibit Bax-induced apoptosis).

Reportable Outcome

All the key research accomplishments are reportable results for the future publication. To publish our findings, we think that further study identifying the minimum element for the binding is necessary. We will continue our investigation to prepare manuscript for submission.